

# Characterization of Insulin-Like Growth Factor-Binding Proteins in the Uterus and Conceptus during Early Conceptus Elongation in Cattle<sup>1</sup>

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## ABSTRACT

As a first step in determining the role that insulin-like growth factor (IGF)-binding proteins (BPs) may have in regulating initial stages of conceptus elongation in cattle, the type and relative abundance of IGFBPs in serum, uterine tissues, and uterine fluid from pregnant and noninseminated cows on Days 13 and 15 postestrus and in Day 15 conceptuses was evaluated. Uterine and serum samples contained IGFBPs 2, 3, 4, and 5 as determined by immunoprecipitation followed by Western ligand blots of precipitates. Compared with those in uterine and serum samples, IGFBPs in conceptuses and conceptus-conditioned culture media were only faintly detectable. The percentage of the total IGF-I binding activity attributed to IGFBP-3 was greater ( $p < 0.05$ ) in myometrium, serum, and uterine fluid ( $> 50\%$ ) than in inter- (40%) and intracaruncular (37%) endometrium. Percentage of total binding attributed to IGFBP-2 was greater ( $p < 0.05$ ) in endometrium and serum ( $\sim 30\%$ ) than in myometrium (16%) and uterine fluid (9%). Binding activity of certain IGFBPs varied due to day of the estrous cycle or due to pregnancy status. Concentrations of IGF-I in serum were greater ( $p < 0.05$ ) in non-pregnant ( $52 \pm 2$  ng/ml) than in pregnant ( $40 \pm 4$  ng/ml) cows. Concentration of IGF-I in uterine fluid did not differ due to pregnancy status or stage of cycle ( $4.4$  pg IGF-I/ $\mu$ g uterine protein). Northern blots revealed mRNAs for IGFBPs 1, 2, 3, 4, and 5 in uterine tissues but not in conceptuses. In situ hybridization indicated that IGFBP-1 mRNA was primarily localized in luminal epithelium of endometrium; IGFBP-2 mRNA was in luminal epithelium and dense stromal cells adjacent to endometrial epithelium; and IGFBP-3 mRNA was in vascular endothelial cells and was more prevalent in myometrium than in endometrium. Tissue specificity and changes in abundance of IGFBPs in the uterus during early conceptus elongation indicate the potential importance of IGFBP regulation of uterine IGFs during this time period.

## INTRODUCTION

In cattle, nearly 20% of pregnancies fail between blastocyst formation (Day 7 of pregnancy) and maternal recognition of pregnancy (Day 16 of pregnancy) [1–6]. Only a small fraction of embryonic deaths are attributable to detectable morphological chromosomal abnormalities [7]; therefore, a substantial portion of embryonic loss probably is due to physiological alterations resulting in discord between the maternal environment and conceptus. The vari-

ation in length observed among conceptuses on a single day is striking, and conceptuses that are delayed developmentally likely are less efficacious at preventing luteolysis than more advanced conceptuses. Thus a potential period for the occurrence of embryonic wastage is the time during which spherical or elliptical conceptuses, generally still less than 1 mm in diameter on Day 13 of pregnancy [8], elongate to more than 100 times that length by Day 16 [9]. Reports of molecular and cellular mechanisms regulating conceptus growth during this stage in the ruminant are virtually absent from the literature.

The insulin-like growth factor (IGF) system is a ubiquitous mitogenic system present in the uterine and/or conceptus environment of many species, including the cow [10–12], sheep [13–16], pig [17–20], horse [21], human [22, 23], and rodent [24–27]. Gene ablation and mutation studies [25, 27, 28] conclusively demonstrated involvement of the IGF system in early conceptus development in rodents. However, basic information concerning the role of the IGF system in regulating uterine physiology and conceptus development in cattle is lacking.

Conceptus growth undoubtedly relies on interactions of growth factors and their receptors. In the IGF system, a third and very critical component exists: IGF-binding proteins (IGFBPs; reviewed in [29] and [30]). The IGFBPs, of which six have been cloned and partially characterized [31], are crucial regulators of IGF function. Bioavailability and interaction of IGF-I and IGF-II with cellular receptors are modulated by these proteins [29, 30, 32]. Therefore, the objectives of this study were to identify and characterize relative abundances of IGFBPs in the uterus and conceptus during the initial stages of conceptus elongation. Western ligand blot analysis was used to compare IGFBP profiles in serum, uterine tissues, and uterine luminal fluid (ULF) on Days 13 and 15 postestrus and in Day 15 conceptuses and conceptus-conditioned culture media. Steady-state levels and localization of IGFBP mRNAs in uterine tissues and conceptuses were evaluated, and concentrations of IGF-I in serum and ULF were measured.

## MATERIALS AND METHODS

### Experiment 1

On Day 13 or 15 postestrus, noninseminated and inseminated pregnant beef cows were subjected to nonsurgical uterine irrigation [33] followed by surgical hemihysterectomy. Eighteen animals were used in a  $2 \times 2$  factorial design (day  $\times$  status) with four noninseminated and five pregnant cows used on each day. Tissues were collected from two groups of cows 3–4 mo apart, with all treatments represented in each group. Conceptuses were collected by insertion of a 24-gauge Foley catheter through the cervix into the proximal half of the uterine horn ipsilateral to the ovary with a corpus luteum. To sample uterine fluid, 22 ml of sterile 0.9% saline was infused through the catheter. The

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uterine horn was massaged gently per rectum, and fluid was recovered. Saline recovered from inseminated cows was examined under a dissecting microscope to determine whether the conceptus was present in this initial sample. Volume of recovered fluid was recorded, and the fluid was centrifuged at  $2000 \times g$  for 15 min to remove debris prior to storage at  $-70^{\circ}\text{C}$  until subsequent evaluation of ULF proteins. The catheter then was retracted to the internal os of the cervix, and at least 500 ml of modified Dulbecco's PBS with 0.1% BSA was infused and collected according to standard embryo recovery procedures [33]. If no embryo was found in either uterine flushing, the cow was replaced if it had been inseminated. Lengths and widths of whole conceptuses and conceptus pieces were measured. A blood sample was collected from each cow, allowed to clot at  $4^{\circ}\text{C}$  for 4–6 h, and centrifuged at  $2000 \times g$  for 15 min. Serum was decanted and stored at  $-70^{\circ}\text{C}$  until analysis of IGFBPs and IGF-I.

Within 1 h of the ULF/embryo collection, anesthesia was induced with 5 g thiopental i.v., and cows were maintained on gas anesthesia (2–5% halothane). The uterine horn ipsilateral to the corpus luteum was exteriorized via midventral incision. The anterior two thirds of the horn was removed surgically and placed on ice. Tissues (caruncle, intercaruncular endometrium, and myometrium) were dissected, frozen immediately in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . In addition, tissue blocks composed of myometrium and caruncular and intercaruncular endometrium were placed in Tissue-Tek OCT embedding medium (Miles Inc., Elkhart, IN) and frozen in liquid nitrogen for subsequent evaluation by in situ hybridization. Serum and ULF samples were analyzed in a single RIA to determine concentrations of IGF-I [34]. Human recombinant IGF-I (DRGO10; Bachem, Torrance, CA) was used as the standard and as the radiolabeled antigen (specific activity was approximately  $130 \mu\text{Ci}/\mu\text{g}$ ). The primary antiserum was UB3-189 (provided by the National Hormone and Pituitary Program, Rockville, MD). The intraassay coefficient of variation was 7.8%.

## Experiment 2

Although conceptuses were collected in experiment 1, the quantity of conceptus tissue obtained was not sufficient to conduct the next experiment. Therefore, four cows were superovulated with 6, 6, 4, 4, 2, 2, 2, and 2 mg FSH-P (Schering, Kenilworth, NJ) i.m. at 12-h intervals beginning between Days 9 and 12 of the estrous cycle. Prostaglandin  $\text{F}_{2\alpha}$  (25 and 12.5 mg, Lutalyse; Upjohn, Kalamazoo, MI) was administered i.m. with the sixth and seventh FSH injections, respectively. Cows were inseminated with frozen-thawed semen 12 and 24 h after estrus was first noted. On Day 15 postestrus, conceptuses were recovered nonsurgically as described above without a preliminary uterine horn irrigation to collect ULF. Upon collection, whole conceptuses and conceptus pieces were measured and washed three times in Dulbecco's PBS + 0.01% BSA. Conceptuses were collected from all 4 cows. A total of 7 intact conceptuses and 13 large conceptus pieces (damaged by the irrigation process) were recovered. Elongated conceptuses are very fragile and easily damaged by the irrigation process, thereby resulting in the pieces collected in this study. Whole conceptuses averaged 8 mm in length and pieces averaged 31 mm in length. Approximately one third of the conceptus tissue from each cow was then washed three times in Dulbecco's PBS, and each piece/conceptus was frozen individually in liquid nitrogen. Remaining concep-

tuses and/or conceptus pieces were cultured individually in 1 ml or 0.33 ml (depending on the length) of chemically defined medium with Eagle's minimum essential medium amino acids, modified as described previously [35], plus 0.01% BSA in 4-well Nunc dishes (Irvine Scientific, Irvine, CA) without a covering of oil. Incubation occurred at  $39^{\circ}\text{C}$  in 5%  $\text{CO}_2$  in air for 3 or 6 h to allow sufficient time for protein secretion. After culture, tissues were washed three times in Dulbecco's PBS and frozen individually. Conditioned media were aspirated, treated with protease inhibitors (1  $\mu\text{M}$  pepstatin A, 1  $\mu\text{M}$  leupeptin, 100  $\mu\text{M}$  PMSF, and 100  $\mu\text{M}$  EDTA), and frozen at  $-70^{\circ}\text{C}$ .

## Analysis of IGFBPs by Western Ligand Blots

To evaluate IGFBPs, tissues from experiments 1 and 2 were homogenized at a concentration of 100 mg tissue/ml ice-cold buffer (0.1% SDS, 1% sodium cholate, 100  $\mu\text{M}$  EDTA, 1  $\mu\text{M}$  leupeptin, 1  $\mu\text{M}$  pepstatin A, and 100  $\mu\text{M}$  PMSF) and centrifuged ( $13\,000 \times g$ ) for 10 min at  $4^{\circ}\text{C}$ . Protein contents of tissue supernatants, uterine fluids, and conceptus-conditioned media (as adjusted to account for protein concentration of unconditioned media) were determined (BCA Protein Assay; Pierce Immunotech, Rockford, IL). Serum (2  $\mu\text{l}$ ) and tissue homogenates (200  $\mu\text{g}$  protein) were diluted 1:5 with gel-loading buffer (62.5 mM Tris base, 2% SDS, 0.02% bromophenol blue, and 10% glycerol, pH 6.8). Uterine fluids and conceptus-conditioned media (200  $\mu\text{g}$  protein) were concentrated by ethanol precipitation and resuspended in 40  $\mu\text{l}$  of the gel-loading buffer. Samples were then subjected to one-dimensional SDS-PAGE under nonreducing conditions [36] through a 4% stacking gel and a 12% separating gel. Proteins were transferred to nitrocellulose membranes, and IGFBPs were detected by ligand blot analysis [34, 37]. Intensity of IGFBP bands was quantified by scanning laser densitometry as previously described [34].

The electrophoresis and transfer apparatus used for the ligand blot procedure had the capacity for 4 gels with 15 lanes per gel. Therefore, all uterine samples from one replicate (i.e., collection date) were processed simultaneously and all membranes from a replicate were exposed on the same piece of film. Within replicate, all samples of the same tissue type (i.e., caruncle, myometrium, endometrium, or ULF) were included on a single gel. Serum samples from the first collection date were stratified by treatment across the 4 gels. Samples from replicate 2 were loaded on an additional pair of gels, each gel containing all samples of two tissue types. For experiment 2, 200  $\mu\text{g}$  of protein from homogenates of individual conceptuses or conceptus pieces ( $n = 14$ ) and conditioned culture media was loaded in separate lanes. Homogenates from conceptus pieces having less than 200  $\mu\text{g}$  protein were combined within treatment (time in culture) and cow before loading. An additional gel containing all four uterine sample types and serum from a single animal was run for illustrative purposes to demonstrate differences in apparent mass of binding proteins among different types of samples (see Fig. 1).

## Identification of IGFBPs by Immunoprecipitation and Western Ligand Blots

Identity of IGFBPs in uterine tissues and fluid, serum, and conceptuses was determined by immunoprecipitation of samples with antisera or antibodies against specific IGFBPs followed by ligand blot analysis of the precipitates. For immunoprecipitation, tissue homogenates and uterine



fluid (200  $\mu$ g protein equivalent) and 3  $\mu$ l of serum + 24  $\mu$ l wash buffer (50 mM Tris, 0.3 M NaCl, 1 mM EDTA, 2% [v:v] Triton X-100, and 0.02% [w:v] NaN<sub>3</sub>, pH 7.4) were combined with 2  $\mu$ l of rabbit anti-human IGFBP-5 antiserum (Upstate Biotechnology, Lake Placid, NY) and incubated overnight at 4°C while rotating on a Roto-Torque (Cole Parmer Instrument Corp., Chicago, IL). The following day, ligand-antibody complexes were precipitated by the addition of 20  $\mu$ l preprecipitated goat anti-rabbit gamma globulin [38]. Samples were incubated 3 h at 4°C with rotation and then centrifuged at 12 000  $\times$  g for 10 min at 4°C. Immunoprecipitation of IGFBP-2 and IGFBP-4 remaining in the supernatant was done sequentially using antisera against IGFBP-2 (rabbit anti-rat IGFBP-2 provided by Nicholas Ling, The Whittier Institute, La Jolla, CA) [39] and IGFBP-4 (rabbit anti-human IGFBP-4; Upstate Biotechnology). Precipitates were washed three times with 0.4 ml wash buffer, dissolved in 40  $\mu$ l gel-loading buffer, and subjected to ligand blot analysis as described above. Precipitated samples were loaded in lanes adjacent to unprecipitated samples to compare locations of bands. Attempts to precipitate IGFBP-1 and IGFBP-6 with a rabbit antiserum against human IGFBP-1 (Upstate Biotechnology) and a rabbit antiserum against rat IGFBP-6 (provided by Nicholas Ling) [39] were not successful.

#### Northern Blot Analyses

Tissues were homogenized in guanidinium thiocyanate, and total RNA was purified through a CsCl gradient by centrifugation [40]. For detection of steady-state levels of mRNA of IGFBPs 1, 2, 3, 4, and 5, total RNA was isolated from uterine tissues collected in experiment 1 and from conceptuses collected from superovulated cows 15 days postestrus according to the procedures described for experiment 2. Conceptuses from within a cow were pooled to yield sufficient quantities of RNA for analysis. In addition, total RNA from uterine tissues of a Day 8–9 uninseminated cow and caruncle, cotyledon, kidney, lung, liver, and fetal liver from a midgestation cow were used as either positive or negative controls. For Northern blots, 15  $\mu$ g of total RNA was denatured in 20  $\mu$ l 50% formamide/2.2 M formaldehyde in single-strength 3-[N-morpholino]propanesulfonic acid (MOPS) buffer (0.04 M MOPS, pH 7, 10 mM sodium acetate, 1 mM EDTA, pH 8) at 65°C for 10 min. Samples were placed on ice, and 5  $\mu$ l of 5-strength sample buffer (0.5% SDS, 0.25% bromophenol blue, 25% glycerol, 25 mM EDTA) was added. Samples were subjected to electrophoresis through a 1.2% agarose-formaldehyde gel and transferred by capillary blotting to nylon membranes (Nytran; Schleicher and Schuell, Keene, NH); RNA was subsequently UV cross-linked to membranes. After linearization of cDNA (bovine IGFBP-1, provided by Dr. R. Renaville, Gembloux, Belgium [41]; rat IGFBPs 2, 4, and 5, provided by Dr. Shunichi Shimasaki, Scripps Research Institute, La Jolla, CA [42]), antisense [<sup>32</sup>P]UTP-labeled riboprobes (cRNA) were generated using SP6, T7, and T3 polymerases (Ambion, Austin, TX). The cDNA for bovine IGFBP-3 (provided by Dr. D. Clemmons, University of North Carolina, Chapel Hill, NC) was labeled with [<sup>32</sup>P]dCTP by random priming (cDNA probe; Gibco, Grand Island, NY). After prehybridization of membranes for 2–4 h at 65°C (cRNA probes) or 42°C (cDNA probe) in hybridization buffer (50% formamide, 250  $\mu$ g/ml boiled salmon sperm DNA, single-strength Denhardt's, 50  $\mu$ g/ml poly A RNA [Sigma Chemical Co., St. Louis, MO], 0.1% SDS,

5-strength SSC [single-strength SSC: 0.15 M NaCl, 0.015 M sodium citrate]), probes (2  $\times$  10<sup>6</sup> cpm/ml) were added and incubated with membranes overnight at 65°C (cRNA probes) or 42°C (cDNA probe). Membranes were washed (cDNA probe: double-strength SSC, 0.1% SDS, for 30 min at 42°C; 0.5-strength SSC, 0.1% SDS, at 50°C for 30 min; and 0.1-strength SSC, 0.1% SDS, at 50°C for 15 min; cRNA probes: twice in double-strength SSC, 0.1% SDS for 10 min at room temperature and twice in 0.2-strength SSC, 0.1% SDS for 30 min at 65°C) and exposed to x-ray film (X-OMAT; Eastman Kodak, Rochester, NY) for 1–5 days at –70°C.

#### In Situ Hybridization of mRNA

Localization of mRNAs for IGFBPs 1, 2, and 3 was performed using <sup>33</sup>P-labeled single-stranded antisense riboprobes that were generated using SP6, T7, or T3 polymerase as described above. The corresponding <sup>33</sup>P-labeled sense strand riboprobes were used as negative controls. Cryosections (10  $\mu$ m) of uterine tissue from 4 cows (1 cow from each stage by status classification) were fixed in 4% paraformaldehyde, washed in double-strength SSC, subjected to proteinase K (1.33  $\mu$ g/ml) digestion, refixed, and washed. After a brief dip in triethanolamine (0.1 M, pH 8.0), tissues were acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, dipped in double-strength SSC, and dehydrated through 70%, 95%, and 100% ethanols. Seventy microliters of denatured probe (2  $\times$  10<sup>7</sup> cpm/ml) in hybridization buffer (60% formamide, 0.36 M NaCl, 12 mM Tris, pH 8.0, 1.2 mM EDTA, 1.2-strength Denhardt's, 12% dextran SO<sub>4</sub>, 0.5 mg/ml tRNA, 0.5 mg/ml poly A) was applied to each slide, and tissues were incubated overnight at 55–60°C. Sections were washed twice in double-strength SSC for 10 min and placed in double-strength SSC containing 20 mg/ml RNase A (Sigma) for 30 min at 37°C. After two more washes in double-strength SSC for 10 min at room temperature, sections were washed stringently in a solution of 0.1-strength SSC, 1 mM EDTA at 55°C for 2 h; this was followed by two additional washes in 0.5-strength SSC for 10 min at room temperature. Tissues were dehydrated through graded alcohols each containing 0.3 M ammonium acetate. Sections were subsequently coated with photographic emulsion (NTB-2; Eastman Kodak), exposed at 4°C for 1–2 wk, developed with Kodak D19, and stained with hematoxylin and eosin.

#### Statistical Analyses

Data from the ligand blot detection of IGFBPs were evaluated by two different approaches. The first approach used the relative quantities (i.e., arbitrary densitometer units) for each individual IGFBP and total IGFBP activity. The second approach used the percentage of the total IGFBP activity that was attributed to each binding protein in a sample. This was calculated by dividing the densitometry units for each individual IGFBP by the sum of the densitometry units for all IGFBPs in the sample, and the values were expressed on a percentage basis. While this second approach is directly influenced by differences in relative quantities of each individual IGFBP, it was used to provide insight into changes or differences in the overall composition of IGFBPs that likely would influence the physiological function of the IGF system due to differences in the affinity of each IGFBP for the IGFs.

The effects of day of estrous cycle or pregnancy (stage) and pregnancy status (status) and the interaction of these

terms on densitometric values for each IGFBP, percentage of total binding attributed to each IGFBP (i.e., composition of total IGFBP activity), and IGF-I concentrations were analyzed separately for each sample type by an ANOVA for a block (replicate) design using the General Linear Models procedures of the Statistical Analysis System [43]. Much of the variation due to replicate is explained by differences in exposure of autoradiographs because ligand blots from the two groups of cows were done independently. Replicate was not significant ( $p > 0.1$ ) in the analysis of percentage of total binding of  $^{125}\text{I}$ -IGF-I by each IGFBP and was eliminated from the model. For analyses of IGFBPs in serum, gel within replicate was initially included in the model and found to be nonsignificant ( $p > 0.1$ ). Data were tested for heterogeneity of variance by Hartley's test and log transformed if appropriate. In such cases, nontransformed means are presented. Except as noted in *Results*, the interaction term (stage  $\times$  status) did not account for a significant amount of the variation in the variables analyzed and was included in the error term. To compare IGFBPs across tissues, actual densitometric values and percentage of total IGFBP activity attributed to individual bands were analyzed in a model containing collection date, stage, status, and tissue. Numbers of animals used for all analyses of IGFBPs were as follows: caruncle, intercaruncular endometrium, and myometrium,  $n = 18$ ; ULF,  $n = 17$ ; serum,  $n = 16$ . For comparisons of IGFBPs across tissues, differences between least-squares means were determined by Tukey's test.

## RESULTS

### Identification of IGFBPs by Immunoprecipitation and Western Ligand Blots

Western ligand blot analysis using  $^{125}\text{I}$ -IGF-I resulted in the detection of six bands (approximate masses of 44, 40, 34, 29–31, 28–29, and 24 kDa) in all uterine (caruncle, intercaruncular endometrium, myometrium, and ULF) and serum samples and a seventh band (approximately 32 kDa) in some samples. Identification of specific binding proteins shown in Figure 1 is summarized from results obtained from samples subjected to immunoprecipitation and ligand blot analysis (not shown). Two glycosylated forms of IGFBP-3 (44 and 40 kDa) have been identified previously in bovine serum and follicular fluid [34, 38, 44]; therefore, identities of these bands were not reconfirmed in the present study. The 34-kDa protein was identified as IGFBP-2. Immunoprecipitation with antibodies against IGFBPs 4 and 5 identified bands at 24 and 28 kDa for IGFBP-4 and 29–31 kDa for IGFBP-5. However, immunoprecipitation with the IGFBP-4 and -5 antibodies failed to precipitate a substantial portion of the binding activity in the 28- to 31-kDa range in comparison to that in samples not subjected to immunoprecipitation. Furthermore, the apparent mass of binding proteins detected between 28 and 31 kDa differed among tissues (Fig. 1). In myometrium and serum, bands in the 28- to 31-kDa range were of slightly greater apparent mass than in caruncle, intercaruncular endometrium, and ULF, which may reflect differences in glycosylation of the same binding protein or may represent different binding proteins. Some binding in this range may be accounted for by IGFBP-1 and/or IGFBP-6. However, attempts to characterize IGFBPs 1 and 6 by immunoprecipitation were not successful.

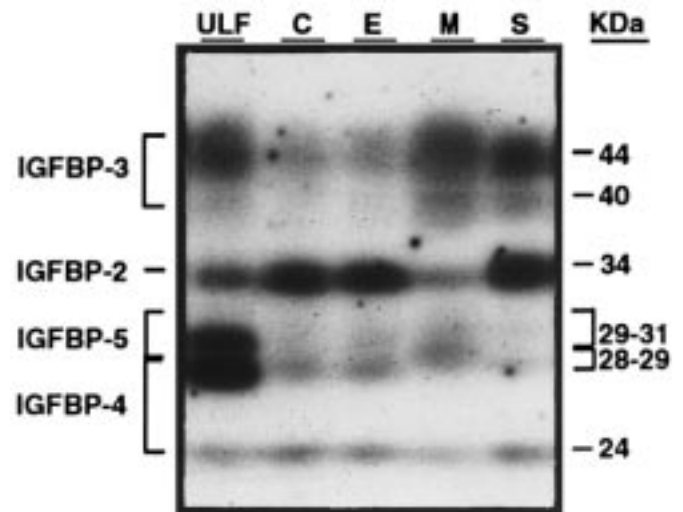


FIG. 1. Western ligand blot of IGFBPs in 200  $\mu\text{g}$  protein from ULF, caruncle (C), intercaruncular endometrium (E), myometrium (M), and 2  $\mu\text{l}$  of serum (S) from a Day 13 pregnant cow. The blot was probed with  $^{125}\text{I}$ -IGF-I. Location of bands for IGFBPs 2, 4, and 5 was determined by ligand blot analysis of immunoprecipitated samples (not shown). Approximate mass of IGFBPs indicated on the right side of figure was extrapolated from molecular weight markers.

### Relative Amounts of IGFBPs in the Various Tissues and Fluids

The relative amounts of individual and total binding activity (i.e., arbitrary densitometer units) differed among sample types when compared on an equivalent total protein concentration basis (Fig. 2). The sum of all binding protein activity (Fig. 2A) was greater ( $p < 0.05$ ) in myometrium than in intercaruncular endometrium, with binding activity in ULF and caruncle being intermediate. Binding activity of IGFBP-2 was greater ( $p < 0.05$ ) in intercaruncular endometrium and caruncle than in myometrium, and was least ( $p < 0.05$ ) in ULF (Fig. 2B). Binding activity of IGFBP-3 was greater ( $p < 0.05$ ) in ULF and myometrium than in intercaruncular endometrium and caruncle (Fig. 2B). Binding activity by proteins in the 28- to 29-kDa and 29- to 31-kDa ranges appeared to be greater in the ULF compared to the uterine tissues (Fig. 2C). However, comparisons of the 28- to 31-kDa IGFBPs across sample types were not performed because the apparent mass of proteins detected in these ranges was not consistent across sample types and thus may reflect different proteins (Fig. 1). Uterine fluid contained less ( $p < 0.05$ ) of the 24-kDa form of IGFBP-4 than myometrium and caruncle but not intercaruncular endometrium, and relative amounts of this protein did not differ ( $p > 0.05$ ) among myometrium, caruncle, and intercaruncular endometrium.

### Composition of the Total IGFBP Activity in the Different Tissues and Fluids

In addition to evaluation of differences in relative amounts (densitometric units) of IGFBPs that were observed in the uterine tissues and fluid (presented in the previous section), data for each IGFBP were expressed as a percentage of total binding activity observed in each sample type to determine whether differences in the equilibrium among IGFBPs in the different tissues and fluids existed. Expressing data as a percentage of the total binding also provided a method to allow comparison of uterine samples

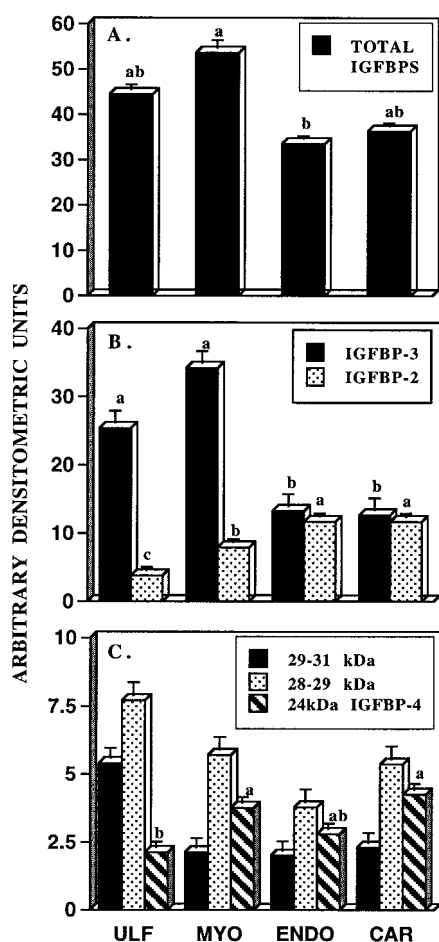


FIG. 2. Binding intensity of  $^{125}\text{I}$ -IGF-I to IGFBPs in 200  $\mu\text{g}$  protein from ULF, myometrium (MYO), intercaruncular endometrium (ENDO), and caruncle (CAR). Values represent least-squares means  $\pm$  SE of arbitrary densitometer units for total binding activity (A), IGFBPs 3 and 2 (B), and smaller-sized IGFBPs (C) of all cows regardless of stage or status. Data were log transformed for analysis. Values without a common superscript for IGFBPs differ ( $p < 0.05$ ) across sample types. Statistical analysis of data for the 28- to 29- or 29- to 31-kDa IGFBPs was not performed because mass and/or identity of proteins within these ranges appear to differ among sample types (see text).

(evaluated on an equivalent total protein basis) to serum (evaluated on a volume basis). Composition of binding activity in caruncular and intercaruncular endometrium was nearly identical (Fig. 3). IGFBP-3 accounted for considerably less ( $p < 0.05$ ) of the total binding protein activity in caruncle (37%) and intercaruncular endometrium (40%) than in myometrium, serum, and ULF. Among these latter three types of samples, IGFBP-3 accounted for a greater ( $p < 0.05$ ) percentage of the total binding activity in myometrium (62%) than in serum (52%); the percentage of total binding activity attributed to IGFBP-3 in ULF was intermediate (56%). The percentage of the total binding activity attributed to IGFBP-2 was similar among caruncle, intercaruncular endometrium, and serum (approximately 30%) and was greater ( $p < 0.05$ ) than in ULF (9%) and myometrium (16%). Binding proteins in the 24- to 31-kDa range accounted for 18–35% of the total binding protein activity in the different types of samples (Fig. 3). However, the percentage of total binding activity accounted for by the individual protein bands in this size range differed among the sample types. Percentage of the total binding activity accounted for by 29- to 31-kDa proteins was 2- to 3-fold

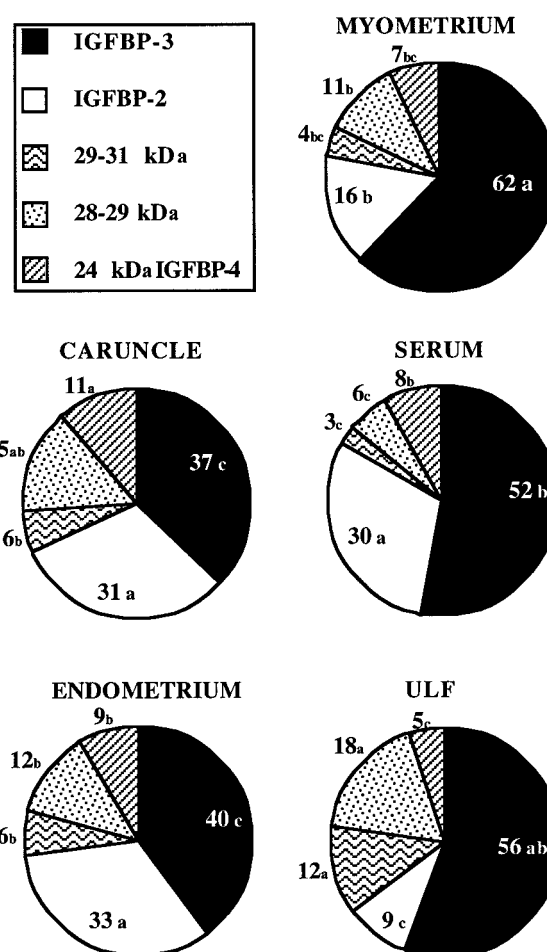


FIG. 3. Composition of total IGFBP activity in the various types of samples. Values represent the percentage of the total binding activity that was accounted for by IGFBPs 3 and 2, 24-kDa IGFBP-4, or binding in the 28- to 29- and 29- to 31-kDa ranges. Values without a common superscript differ ( $p < 0.05$ ) among tissues and fluids.

greater ( $p < 0.05$ ) in ULF (12%) than in other samples. Proteins in the 28- to 29-kDa range accounted for a greater percentage ( $p < 0.05$ ) of the total binding activity in ULF (18%) and caruncle (15%) than in serum (6%). In intercaruncular endometrium (12%) and myometrium (11%), percentage of total binding accounted for by proteins in this range was less ( $p < 0.05$ ) than in ULF, but greater ( $p < 0.05$ ) than in serum. The percentage of total binding activity accounted for by the 24-kDa form of IGFBP-4 was greatest ( $p < 0.05$ ) in caruncle (11%) compared to all other sample types and less ( $p < 0.05$ ) in ULF (5%) compared to serum (8%) and intercaruncular endometrium (9%).

#### Effects of Stage of Cycle and Pregnancy Status on Relative Amounts of IGFBPs

Differences in relative amounts of IGFBPs (i.e., densitometric values) due to stage or status that were statistically significant are presented in Table 1. Specific IGFBPs not presented in Table 1 were not ( $p > 0.1$ ) influenced by stage or status. In intercaruncular endometrium, binding of  $^{125}\text{I}$ -IGF-I by 29- to 31-kDa proteins was greater ( $p < 0.05$ ) in pregnant than in nonpregnant cows, and binding of  $^{125}\text{I}$ -IGF-I by proteins in the 28- to 29-kDa range was greater ( $p < 0.05$ ) on Day 15 than on Day 13. In myometrium, abundance of IGFBP-2 increased ( $p < 0.05$ ) between Days



TABLE 1. List of IGFBPs that were influenced ( $p < 0.10$ ) by stage of cycle (Day 13 vs. Day 15) or pregnancy status (not pregnant [NP] vs. pregnant [P]).\*

| Tissue      | IGFBP                       | Day 13 NP      | Day 13 P       | Day 15 NP      | Day 15 P       |
|-------------|-----------------------------|----------------|----------------|----------------|----------------|
| Endometrium | 29-31 kDa <sup>a</sup>      | 1.1 $\pm$ 0.6  | 2.4 $\pm$ 0.5  | 2.0 $\pm$ 0.6  | 2.8 $\pm$ 0.5  |
|             | 28-29 kDa <sup>c</sup>      | 3.0 $\pm$ 0.7  | 2.9 $\pm$ 0.6  | 4.5 $\pm$ 0.7  | 5.9 $\pm$ 0.6  |
| Myometrium  | 34 kDa IGFBP-2 <sup>c</sup> | 5.3 $\pm$ 2.0  | 5.7 $\pm$ 1.7  | 11.9 $\pm$ 2.0 | 8.7 $\pm$ 1.7  |
|             | 29-31 kDa <sup>b</sup>      | 1.6 $\pm$ 0.4  | 2.4 $\pm$ 0.3  | 2.1 $\pm$ 0.4  | 2.1 $\pm$ 0.4  |
| ULF         | 44 kDa IGFBP-3 <sup>b</sup> | 24.2 $\pm$ 5.5 | 14.9 $\pm$ 4.8 | 27.2 $\pm$ 5.5 | 17.5 $\pm$ 5.3 |
| Caruncle    | 40 kDa IGFBP-3 <sup>d</sup> | 1.6 $\pm$ 0.6  | 2.7 $\pm$ 0.5  | 2.8 $\pm$ 0.6  | 1.9 $\pm$ 0.5  |

\* Values shown are relative amounts of IGFBP activity (least-squares means  $\pm$  SE for nontransformed arbitrary densitometric units) per 200  $\mu$ g protein from uterine tissues and fluid; data were log transformed before statistical analysis to correct for heterogeneity of variance.

<sup>a</sup> Status effect  $p < 0.05$ .

<sup>b</sup> Status effect  $p < 0.10$ .

<sup>c</sup> Stage effect  $p < 0.05$ .

<sup>d</sup> Stage  $\times$  status effect  $p < 0.10$ .

13 and 15, and binding of <sup>125</sup>I-IGF-I by 29- to 31-kDa IGFBPs tended to be greater ( $p < 0.1$ ) in pregnant than in nonpregnant cows. Other trends noted were greater amounts of the 44-kDa form of IGFBP-3 in ULF of nonpregnant animals ( $p < 0.1$ ) as compared to pregnant animals and an interaction between stage and status for the 40-kDa form of IGFBP-3 in caruncle. Day 15 nonpregnant animals had more ( $p < 0.1$ ) of the 40-kDa form of IGFBP-3 in the caruncle than Day 13 nonpregnant cows, but this effect was not evident in pregnant animals.

#### Effects of Stage of Cycle and Pregnancy Status on IGFBP Composition

Effects of stage of the estrous cycle and pregnancy status on composition of the total binding activity within tissues are shown in Table 2. Percentage of total binding activity attributed to proteins in the 28- to 29-kDa range was greater in caruncle, myometrium ( $p < 0.05$ ), and intercaruncular endometrium ( $p < 0.1$ ) of pregnant animals than in nonpregnant animals. In intercaruncular endometrium, percentage of total binding activity accounted for by proteins in this range also increased ( $p < 0.05$ ) from Days 13 to 15. Percentage of total binding activity accounted for by 29- to 31-kDa proteins in the intercaruncular endometrium was twofold higher ( $p < 0.05$ ) in pregnant than in nonpregnant animals, whereas percentage of the total binding attributed to the 44-kDa form of IGFBP-3 in the intercaruncular endometrium was less ( $p < 0.05$ ) in nonpregnant than in pregnant animals. Percentage of total binding activity accounted for by 44-kDa IGFBP-3 and the 24-kDa form of IGFBP-4 tended ( $p < 0.1$ ) to be greater in the intercaruncular en-

dometrium of Day 13 animals than Day 15 animals. In serum, percentage of total binding attributed to IGFBP-3 was greater ( $p < 0.05$ ), whereas percentage of total binding activity attributed to IGFBP-2 was less ( $p = 0.05$ ), in nonpregnant than in pregnant animals. In ULF, the percentage of total binding activity attributed to the 24-kDa form of IGFBP-4 tended to be greater ( $p < 0.1$ ) in nonpregnant than in pregnant cows. Effects of stage by status interactions on composition of the total IGFBP activity were not significant ( $p > 0.1$ ) in any tissue.

#### IGFBPs in Conceptuses and Conceptus-Conditioned Media

Conceptuses collected nonsurgically from unsuperovulated cows on Days 13 and 15 in experiment 1 of this study and preliminary experiments differed in average surface area by 36-fold [8]. Day 13 conceptuses ( $n = 12$ ) averaged ( $\pm$  SD) 0.8  $\pm$  0.3 mm in length with a mean ( $\pm$  SD) surface area of 1.5  $\pm$  1.0 mm<sup>2</sup>, while Day 15 conceptuses ( $n = 12$ ) averaged 20.7  $\pm$  13.8 mm in length with a mean surface area of 54.1  $\pm$  32.0 mm<sup>2</sup>. When compared to uterine samples, Day 15 conceptuses and conceptus-conditioned media (from experiment 2) were almost devoid of any IGFBP activity. In conceptuses, IGFBPs were not detected in 10 out of 14 samples evaluated by ligand blot, and only very faint bands of IGFBP-2 were detected in the 4 samples with IGFBP activity. In conditioned media, very faint bands of IGFBP-3 and/or IGFBP-2 were observed in 7 out of 11 samples, and the remaining 4 samples did not contain detectable IGFBP activity. Detection of IGFBPs in conceptus tissue and conditioned medium required a longer

TABLE 2. Effects of stage of cycle (Day 13 vs. Day 15) or pregnancy status (not pregnant [NP] vs. pregnant [P]) on percentage of total IGFBP activity (least-squares means  $\pm$  SE) attributed to individual IGFBPs in uterine tissues, ULF, and serum.\*

| Tissue      | IGFBP                            | Day 13 NP      | Day 13 P       | Day 15 NP      | Day 15 P       |
|-------------|----------------------------------|----------------|----------------|----------------|----------------|
| Endometrium | 44 kDa IGFBP-3 <sup>ad</sup>     | 41.2 $\pm$ 3.2 | 34.5 $\pm$ 2.9 | 35.0 $\pm$ 3.2 | 28.9 $\pm$ 2.9 |
|             | 29-31 kDa <sup>a</sup>           | 3.3 $\pm$ 1.8  | 8.1 $\pm$ 1.7  | 4.7 $\pm$ 1.8  | 8.3 $\pm$ 1.7  |
|             | 28-29 kDa <sup>ab</sup>          | 9.2 $\pm$ 2.0  | 9.5 $\pm$ 1.8  | 10.8 $\pm$ 2.0 | 17.6 $\pm$ 1.8 |
|             | 24 kDa IGFBP-4 <sup>d</sup>      | 9.6 $\pm$ 1.3  | 9.9 $\pm$ 1.2  | 7.4 $\pm$ 1.3  | 7.4 $\pm$ 1.2  |
| Myometrium  | 28-29 kDa <sup>a</sup>           | 8.9 $\pm$ 1.5  | 13.4 $\pm$ 1.3 | 8.9 $\pm$ 1.5  | 11.4 $\pm$ 1.3 |
| Caruncle    | 28-29 kDa <sup>a</sup>           | 12.8 $\pm$ 1.5 | 15.8 $\pm$ 1.3 | 13.1 $\pm$ 1.5 | 16.5 $\pm$ 1.3 |
| ULF         | 24 kDa IGFBP-4 <sup>d</sup>      | 5.4 $\pm$ 1.3  | 6.8 $\pm$ 1.1  | 3.4 $\pm$ 1.3  | 4.2 $\pm$ 1.3  |
| Serum       | 44 kDa IGFBP-3 <sup>b</sup>      | 48.7 $\pm$ 4.5 | 35.9 $\pm$ 4.1 | 45.3 $\pm$ 5.2 | 41.5 $\pm$ 4.5 |
|             | 34 kDa IGFBP-2 <sup>b</sup>      | 23.7 $\pm$ 4.1 | 35.4 $\pm$ 3.7 | 26.7 $\pm$ 4.8 | 32.9 $\pm$ 4.1 |
|             | IGFBP-3 (44+40 kDa) <sup>b</sup> | 60.0 $\pm$ 4.6 | 45.0 $\pm$ 4.1 | 58.5 $\pm$ 5.3 | 51.8 $\pm$ 4.6 |

\* Data not shown if  $p > 0.1$  for effects of stage, status, or stage  $\times$  status.

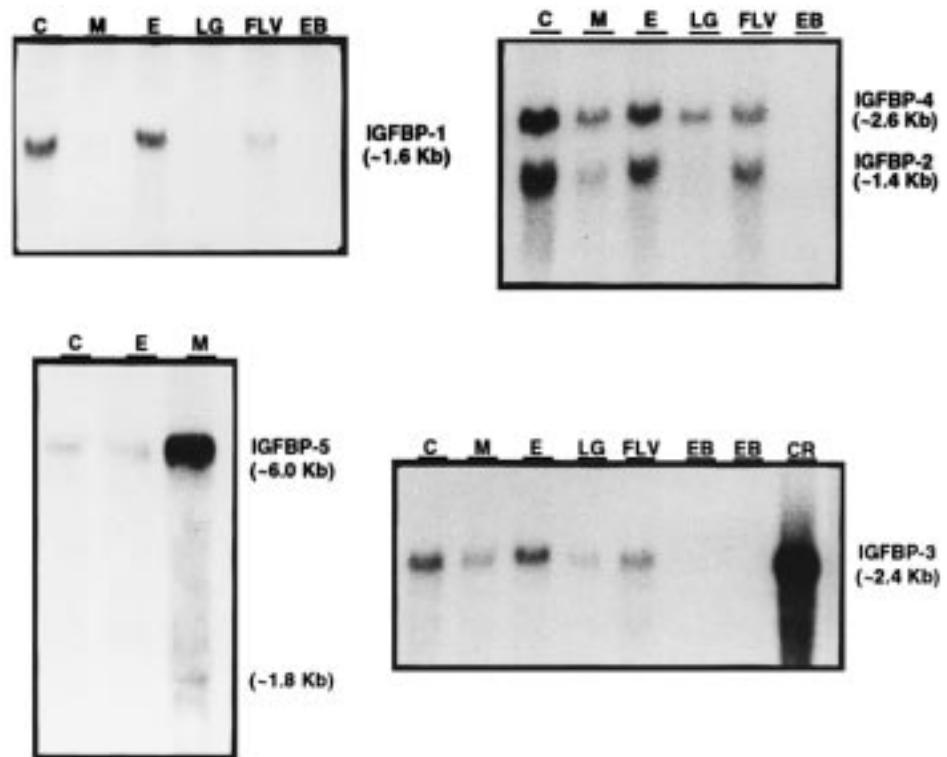
<sup>a</sup> Status effect  $p < 0.05$ .

<sup>b</sup> Status effect  $p < 0.10$ .

<sup>c</sup> Stage effect  $p < 0.05$ .

<sup>d</sup> Stage effect  $p < 0.10$ .

FIG. 4. Northern blots of IGFBPs 1, 2, 3, 4, and 5 mRNAs using 15  $\mu$ g total RNA from caruncle (C), myometrium (M), intercaruncular endometrium (E) and conceptuses (EB). Additional tissues used as positive or negative controls included lung (LG), fetal liver (FLV), and midgestation caruncle (CR). Transcript sizes were approximated by comparison of band locations to 28S and 18S ribosomal RNA bands.



film exposure time than was used for analysis of uterine samples. Because bands were not consistently detected and detection required longer exposure, densitometric analysis was not performed on conceptus-derived samples.

#### Concentrations of IGF-I in Serum and ULF

Concentrations of IGF-I in serum on Days 13 and 15 of the cycle were greater ( $p < 0.05$ ) in nonpregnant cows ( $51.9 \pm 4.2$  ng/ml;  $n = 8$ ) than in pregnant cows ( $39.5 \pm 3.6$  ng/ml;  $n = 10$ ). No effect due to stage was detected. Neither pregnancy status nor day affected concentration of IGF-I in ULF when data were normalized to a constant amount of protein in the sample. Mean concentration of IGF-I in ULF was 4.4 pg/ $\mu$ g protein. Stage by status interactions were not detected for either sample type.

#### Northern Blot Analyses

Steady-state levels of IGFBPs 1, 2, 3, 4, and 5 mRNAs were detected in caruncle, intercaruncular endometrium, and myometrium by Northern blots but not in conceptuses (Fig. 4). Ethidium bromide staining of gels to visualize 28S and 18S ribosomal bands and sequential probing of membranes for IGFBPs provided evidence of RNA integrity. Messenger RNA for IGFBP-1 (approximately 1.4 kilobases [kb]) was readily detected in intercaruncular endometrium and caruncle but was nondetectable in myometrium from 3 out of 5 cows analyzed. Messenger RNAs for IGFBP-2 (1.6 kb), IGFBP-3 (2.4 kb), and IGFBP-4 (2.6 kb) were detected in all three uterine tissues (Fig. 4). Transcripts of 6 kb and 1.8 kb IGFBP-5 mRNA were detected in each uterine tissue from cows on Days 13 and 15 of the cycle. The autoradiograph of IGFBP-5 mRNA pictured in Figure 4 was from a Northern blot using uterine tissues from a cow Day 8–9 postestrus because clarity of bands on this Northern blot was superior to that on Northern blots of tissues from Day

13 or 15 cows that had been probed for other IGFBPs before IGFBP-5.

#### In Situ Hybridization of mRNA

Evidence for tissue-specific expression of IGFBPs 1, 2, and 3 mRNAs was provided by results from in situ hybridization (Fig. 5). Abundant expression of IGFBP-1 mRNA was limited to the luminal epithelium of endometrium and was detected in both Day 13 nonpregnant and Day 15 pregnant animals. Messenger RNA for IGFBP-2 was localized to the luminal epithelium and dense stromal cells adjacent to the endometrial epithelium. Specific hybridization to IGFBP-3 mRNA occurred in the vascular endothelial cells and was more evident in myometrium than in endometrium (Fig. 5). Attempts to localize expression of IGFBP-4 and -5 mRNA using the rodent probes described in *Materials and Methods* were not successful. Hybridization was almost completely absent after treatment with RNase, and sections were not sufficiently free of nonspecific binding when RNase was omitted from the protocol. Possibly these limitations may be overcome with the use of homologous probes, because in situ analysis of rodent uterine tissue with these rodent probes has been previously validated [42]. Transcripts for IGFBPs were not detected in the limited number of conceptus samples available for analysis ( $n \leq 2$  samples evaluated per probe).

#### DISCUSSION

The present research demonstrated that IGFBPs are located and synthesized in the caruncle, intercaruncular endometrium, and myometrium and are present in the uterine lumen of cows on Days 13 and 15 postestrus, when conceptus elongation begins. In comparison to findings in uterine samples and serum, only trace amounts of IGFBPs were detected in homogenates of Day 15 conceptuses, and tran-

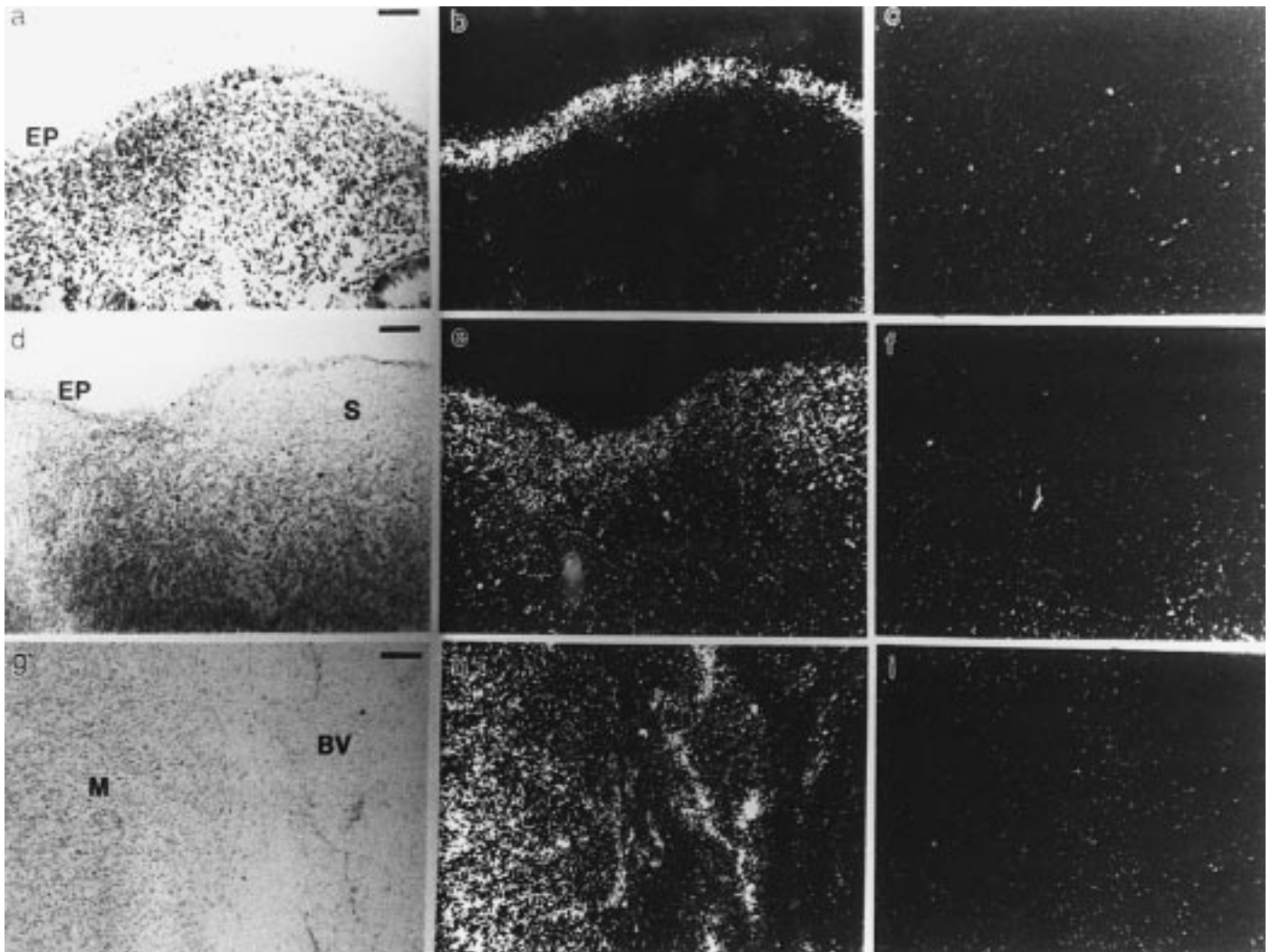


FIG. 5. In situ hybridization of mRNA for IGFBP-1 (top), IGFBP-2 (middle), and IGFBP-3 (bottom) in bovine uterus on Day 15 postestrus. Left (a, d, g): brightfield micrographs. Center (b, e, h): darkfield micrographs of sections hybridized with  $^{33}\text{P}$ -labeled antisense cRNA probes. Right (c, f, i): darkfield micrographs of sections hybridized with  $^{33}\text{P}$ -labeled sense cRNA probes. Localization of IGFBP-1 mRNA was primarily in the luminal epithelium (EP); IGFBP-2 mRNA was in the luminal epithelium and dense endometrial stroma (S); and IGFBP-3 mRNA was localized to vascular endothelial cells of blood vessels (BV) and myometrium (M). Bar = 75  $\mu\text{m}$ .

scripts for IGFBP mRNAs were detected in the uterine samples but not in conceptuses. Therefore, the relatively small quantities of IGFBPs detected in the conceptus may be of uterine origin. Alternatively, the relative proportion of embryonic versus trophoblastic tissue contained in a given sample may have contributed to the inconclusive detection of IGFBP in the conceptus samples. The apparent paucity of IGFBPs in conceptuses compared to uterine samples provides evidence that IGFBP regulation of IGFs may be primarily under maternal rather than conceptus regulation in cattle during this period. This interpretation may require further qualification pending verification of the potential production of IGFBPs by the embryonic disk.

Results from the present study indicate that differences in amounts of IGFBPs (Fig. 2) and the equilibrium among the various IGFBPs (Fig. 3) are regulated in a tissue-specific manner. Profiles of IGFBPs in caruncle and intercaruncular endometrium were very similar, but profiles in these tissues differed markedly from those observed in the myometrium, serum, and ULF. The similarity among IGFBP profiles in caruncular and intercaruncular endometrial tissues may be due to common mechanisms in regu-

lation of these proteins or due to difficulty in absolute separation of glandular and nonglandular material, as noted previously [45]. In any case, caruncular and intercaruncular endometrial tissue contained greater IGFBP-2 and less IGFBP-3 binding activity than did myometrial tissue or ULF. Tissue differences in the levels of these proteins may be due in part to differences in local production. As demonstrated by in situ hybridization, transcripts for IGFBP-2 were most abundant in the luminal epithelium and dense stromal cells immediately adjacent to the luminal epithelium, whereas IGFBP-3 transcripts were most abundant in the vascular endothelial cells and myometrium. Kirby and coworkers [12] found that levels of IGFBP-2 mRNA were greater in extracts of bovine endometrium than in myometrium and that IGFBP-3 mRNA was more abundant in myometrium than in endometrium. Therefore, differences in tissue-specific synthesis of these proteins may be of physiological importance.

When compared to results from other species, the localization of IGFBP-2 mRNA in bovine uteri of the present study was similar to that observed in rat [42] and pig uteri [19] but differed from that observed in human uterine tis-



sue, where diffuse homogeneous expression was observed throughout the stromal compartment [23]. In contrast, localization of IGFBP-3 mRNA in the present study more closely resembled that in the human [23] than in the rat [42]. In uterine tissue of ewes, IGFBP-2 mRNA was not detected by *in situ* hybridization until Day 29 of gestation, when intense hybridization was localized to the dense stroma adjacent to the luminal epithelium. Hybridization to IGFBP-3 mRNA was greatest in the luminal epithelium, moderate in the myometrium, and low in the endometrium during Days 13–15 of gestation [16]. Collectively, these studies provide evidence that sites of expression of IGFBP-2 and -3 in the uterus may differ among species.

Levels of IGFBP-2 in myometrium increased between Days 13 and 15. Although IGFBP-2 activity in endometrium did not differ due to stage of the estrous cycle or pregnancy status when evaluated on Days 13 and 15 in the present study, an increase in IGFBP-2 mRNA in the endometrium from Days 15 to 18 postestrus, coincident with conceptus elongation, has been reported in cattle [10]. In pigs, levels of IGFBP-2 mRNA in the endometrium increased from Days 12 to 18 postestrus, and the increase was more dramatic in pregnant pigs. The potential biological actions of IGFBP-2 in the uterus remain to be substantiated. IGFBP-2 contains an extracellular matrix recognition site (i.e., an arginine-glycine-aspartic acid sequence) [31]. Binding of IGFBP-2 to the extracellular matrix in the uterus may act to increase local concentrations of IGFs within this tissue or to enhance transport of locally produced or circulating IGFs across this tissue for secretion into the uterine lumen, as proposed previously for other physiological systems [46]. Interaction of IGFBP-2 with the extracellular matrix could account for the difference in quantities of IGFBP-2 observed between uterine tissues and ULF. These results provide evidence that IGFBP-2 may have a modulatory role in uterine function during conceptus development.

In the present study, pregnancy was associated with reduced amounts of IGFBP-3 in ULF and with a decrease in the proportion of the total IGF binding activity that IGFBP-3 accounted for in the endometrium and serum. Because IGFBP-3 is the most abundant IGFBP in serum, existing at a concentration of 3–5  $\mu\text{g/ml}$  in humans [47], a portion of the IGFBP-3 detected in uterine tissues and ULF may be serum derived. However, detection of IGFBP-3 mRNA in myometrium provides evidence for local production of IGFBP-3. Perhaps myometrial production of IGFBP-3 has a role in regulating transport of circulating IGFs into the myometrium and subsequent distribution of IGFs to the local surrounding tissues or into the ULF. In addition, specific cellular receptors for IGFBP-3 have been reported [48], and direct actions of IGFBP-3, independent of IGF-I, have been observed on cellular function *in vitro* [49, 50]. On the basis of these observations, substantial research will be needed to determine the physiological role that IGFBP-3 may have in modulating uterine function.

As was observed with IGFBP-2 and -3, the abundance of and proportion of total IGF binding activity attributed to proteins in the 28- to 31-kDa range also differed among sample types. Most notable was the finding that ULF contained greater IGF binding capacity by the 28- to 31-kDa proteins as compared to all other samples types. In addition, the apparent mass of proteins in the 28- to 31-kDa range appeared to be smaller in intercaruncular endometrium, caruncle, and ULF than in myometrium and serum (Fig. 1), indicating potential differences in the types of IGFBPs ob-

served in the various samples. Antibodies to bovine IGFBP-1 were not available. However, transcripts for IGFBP-1 were localized in the luminal epithelium in the present study, and the mass of IGFBP-1 (29–31 kDa) in other species [51] indicates that IGFBP-1 may account for a substantial portion of the 29- to 31-kDa IGFBPs observed in endometrium, caruncle, and ULF. Assuming that IGFBP-1 accounts for some of the 29- to 31-kDa binding protein activity detected in uterine samples, the observed increase in abundance (Table 1) and/or proportion of total binding activity accounted for by this protein (Table 2) due to pregnancy and/or stage would be consistent with the IGFBP-1 production reported for other species. In primates, mRNA for IGFBP-1 was shown to be differentially expressed in secretory endometrium, and IGFBP-1 was the major IGFBP produced by decidualized endometrial stroma under the influence of progesterone during pregnancy [23, 52, 53]. In rats, IGFBP-1 was localized to the luminal and glandular epithelial cells, and levels of mRNA for this protein fluctuated with stage of the cycle [54]. In the sheep uterus, presence of IGFBP-1 was limited to luminal epithelium of pregnant animals between Days 10 and 16 postestrus [55]. In primates, IGFBP-1 may have a role in trophoblast invasion of the endometrium and the decidualization process [56]. However, placentation in ruminants differs in that uterine cells do not undergo decidualization. Therefore, in ruminants, IGFBP-1 may be involved in remodeling of uterine tissues during the luteal phase leading to the impending process of uterine conceptus apposition and eventual implantation. Because mRNA transcripts for IGFBP-1 were not detected in midgestational caruncle and cotyledon in the present study, developmental regulation of this binding protein suggests an important function during early gestation. In any case, the quantities of proteins in the 28- to 31-kDa range changed across tissues; therefore, identification of these proteins is crucial for further understanding of the system.

In the present study, pregnant cows had lower serum concentrations of IGF-I than nonpregnant cows. However, concentration of IGF-I in ULF did not differ due to pregnancy status when data were normalized to a microgram ULF protein basis. While this normalization may introduce enough variation to mask any existing differences due to stage or status, the assumption was made that protein content of uterine fluid ( $10.1 \text{ mg} \pm 0.58$ ) is independent of days postestrus and pregnancy status [45] and varies minimally between Days 13 and 15 [57, 58]. When evaluated between Days 0 and 18 in cattle, total content of IGF-I and IGF-II in uterine flushings was extremely variable among days and not significantly influenced by pregnancy status [10]. However, levels of mRNA for IGF-I in uterine and ovarian tissues were greater in pregnant than nonpregnant cows on Day 17 of the cycle [12]. In contrast, uterine fluid from nonpregnant sheep on Day 16 postestrus had approximately fivefold more IGF-I than in that from pregnant sheep [13], and concentrations of uterine IGF-I mRNA were lower in pregnant ewes than in ewes at estrus [16]. Additional research will be needed to provide explanations for the apparent inconsistencies in the results from these different studies.

Differences in IGFBPs observed between Days 13 and 15 or between pregnant and nonpregnant animals at these specific days of the cycle are consistent with a role of the IGF system in modulating conceptus growth, but conclusions supporting this hypothesis remain to be substantiated. Because many parameters were analyzed and animal num-

bers were limited, interpretation of results regarding stage and status should be made with caution. Except for information gained through *in vitro* conceptus culture experiments [59, 60] and gene mutation studies in rodents [25, 27, 28], conclusions linking the IGF system and conceptus development are predominantly based on descriptive studies and correlative data [17, 19, 20, 45].

In summary, IGFBP activity in uterine tissues and uterine fluid differed due to day postestrus and pregnancy status, coinciding with the onset of conceptus elongation. The relative abundance of uterine IGFBPs differed in a tissue-specific manner, and the proportion that each individual IGFBP contributed to the total  $^{125}\text{I}$ -IGF-I binding activity differed markedly among the tissues analyzed. During elongation, IGFBPs were scarcely detectable in bovine conceptuses as compared to uterine tissues. The complexity of the IGF system, composed of two ligands (IGF-I and IGF-II), at least six binding proteins, two receptors, and proteases that regulate the IGFBPs, makes it an extremely difficult system to evaluate. IGFBPs can either inhibit or enhance IGF action depending on tissue type, concentration, pH, and degree of posttranslational modification [61, 62]. Therefore, the physiological relevance of the present results concerning actions of IGFBPs in altering uterine environment is speculative at the present time. However, results reported here provide a solid foundation for further work to determine the role of the IGF system in bovine conceptus growth.

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